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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12P 19/26, 19/18, 19/04, C12N 11/04, 11/12, A61K 31/715, 31/735, A61L 31/00, C08L 1/02, 5/00, 5/08, 5/04, A61L 27/00, 29/00

A2

(11) International Publication Number: **WO 95/24497**

(43) International Publication Date: 14 September 1995 (14.09.95)

(21) International Application Number: PCT/EP95/00935

(22) International Filing Date: 10 March 1995 (10.03.95)

(30) Priority Data:
PD94000042 11 March 1994 (11.03.94) IT

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(81) Designated States: AM, AT (Utility model), AU, BB, BG, BR, BY, CA, CH, CN, CZ, CZ (Utility model), DE (Utility model), DK (Utility model), EE, ES (Utility model), FI, FI (Utility model), GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ENZYMATIC PREPARATION OF POLYSACCHARIDES

(57) Abstract

Provided are *in vitro* processes for the production of polysaccharides such as hyaluronic acid, cellulose, polymannuronan, chitin, etc. These processes are carried out under reaction conditions that permit the recycling of nucleotide-phosphates required for the formation of sugar nucleotide precursors employed in the synthesis of these polysaccharides. Also provided are polysaccharides produced via these processes, and pharmaceutical and cosmetic compositions containing these polysaccharides.

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ENZYMATIC PREPARATION OF POLYSACCHARIDES

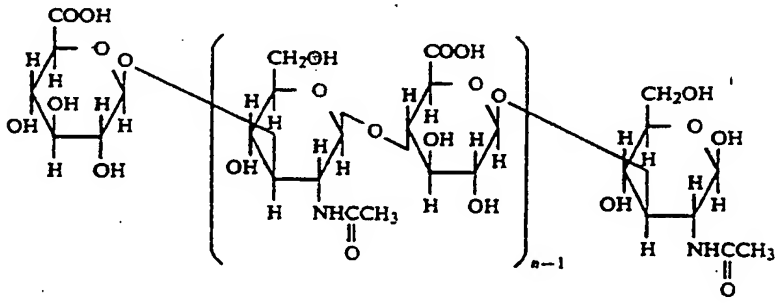
BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a process for the preparation of polysaccharides such as hyaluronic acid (HA) by enzymatic synthesis *in vitro* using a purified protein fraction containing hyaluronate synthase (HAS).

Description of Related Art

Hyaluronic acid is a naturally occurring linear polysaccharide composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine linked by β-1-3 and β-1-4 glycosidic bonds, as shown below:



HA is present in all soft tissues of higher organisms, and in particularly high concentrations in the synovial fluid and vitreous humour of the eye (Laurent et al. (1991) Adv. Drug Deliv. Rev. 7:237). In addition to fulfilling structural roles related to its lubricating and water-retaining properties, evidence is mounting that HA also plays an important role in a number of biological processes such as cell motility and cell-cell interactions (see Laurent et al. (1993) FASEB J. 6:2397 and Knudson et al. (1993) FASEB J. 7:1233 for recent reviews).

Since the discovery of HA, much attention has focussed on the possible biomedical applications of highly purified HA fractions. Numerous studies have proven that HA isolated from various sources has an identical chemical structure. Therefore, since it is already present in the human body, exogenously applied HA of sufficient purity is highly biocompatible (i.e., it does not provoke adverse host reactions) and completely biodegradable by natural catabolic pathways. Solutions of purified HA of high molecular weight are extremely viscous and exhibit very interesting rheological behaviour.

As a consequence of the above properties, HA has been widely exploited in the fields of viscosupplementation and viscosurgery (Laurent et al. (1993) FASEB J. 6:2397). Widespread exploitation of HA in other fields where its natural properties might have rendered it suitable has been limited by the fact that unmodified HA exists only in the form of an aqueous gel and has a short residence time upon administration. Therefore, much attention has been focussed on obtaining chemical derivatives of HA which would maintain the biocompatibility of the parent molecule while allowing it to be processed into products for use in areas such as drug delivery and tissue repair (Rastrelli et al.

(1990) Clinical Implant Materials 199; Balazs et al. (1991) Blood Coagulation Fibrinolysis 2:173).

Sources of Hyaluronic Acid

In order to fulfill the raw material requirements for products for the biomedical applications described above, the identification of dependable and economically viable sources of HA has been an industrial priority.

Initially, attention was focussed on the extraction of HA from animal tissues. Numerous tissue sources, including umbilical cord, skin and rooster combs, have been evaluated. Subsequently, rooster comb HA became the most widely used and traditionally accepted source both from an industrial and regulatory point of view. However, there are certain drawbacks to dependence on this source of HA. High molecular weight material is difficult and costly to isolate due to the fact that the HA is complexed with proteoglycans. Additionally, animal-sourced materials for biomedical applications are coming under increasingly stringent regulatory control due to the fear of contamination with both conventional and unconventional viral agents. Finally, if HA-based products become commonly used in sectors such as drug delivery and tissue repair, predictions indicate that rooster comb supplies will be insufficient to meet the demand for HA. Therefore, attention has turned in recent years to the identification of alternative HA sources.

Lancefield's group A and C streptococci, which are human and animal pathogens, respectively, produce HA and have been exploited for the development of industrial-scale fermentation processes (for example, US patents 4,784,990 and 4,517,295). The equivalence of streptococcal and rooster comb HA has been demonstrated, and the former has now been accepted from a regulatory point of view. Supplies of HA from streptococcal fermentation are theoretically limitless, and no fears

of seasonal fluctuations or batch-to-batch variations exist if a tightly controlled process is used. The HA is produced as a capsule of high molecular weight material which is easily separated from the micro-organisms, although extensive purification is still necessary in order to obtain material of sufficient purity. The possibility of contamination with viral agents is obviously not a major concern, and the use of non-pathogenic mutants in the industrial processes ensures the absence of other toxic impurities.

An added advantage of using streptococci, which has become apparent in recent years, is that recombinant DNA technology can be applied to these organisms. Therefore, the tools of molecular biology can be exploited to facilitate the development of a clear understanding of the molecular mechanisms of HA synthesis and to allow intervention by genetic engineering to modulate this process.

HA Biosynthetic Pathway

An important step in opening up the possibilities of genetic engineering is a definition of the HA biosynthetic pathway. Although no detailed genetic work has been carried out to identify each step in the biosynthetic pathway in streptococci, the succession of biochemical events can be put together based on knowledge of the biosynthesis of the two UDP-sugar precursors of HA gained from studies carried out in other organisms (Brede et al. (1991) J. Bacteriol. 173:7042; Mengin-Lecreuix et al. (1993) J. Bacteriol. 175:6150) and from some limited streptococcal studies (Matsubara et al. (1991) Chem. Pharm. Bull. 39:2446).

The proposed biosynthetic pathway for HA is shown in Figure 1. The precursors, UDP-GlcA and UDP-NAcGlc, are synthesized as side reactions of the glycolytic pathway starting from glucose-6-phosphate and fructose-6-phosphate, respectively. Experiments carried out with

radioactive glucose (O'Regan, unpublished data) have shown that about 5-7% of the glucose in the streptococcal culture medium is converted to HA. Metabolic engineering approaches designed to increase the flux towards HA could be fruitful in terms of obtaining yields that are more industrially viable.

Hyaluronic Acid Synthase

Much attention has been focussed on hyaluronic acid synthase as a key enzyme in the biosynthetic pathway of HA and an essential element in developing an understanding of the mechanism of synthesis.

HA synthase is located in the plasma membrane. This has been shown by a number of different workers using various approaches. Markovitz and Dorfman ((1962) J. Biol. Chem. 238:273) first synthesized HA using streptococcal membranes. Prehm ((1983) Biochem J. 211:181 and 191) demonstrated the extracellular growth of the HA chain and that HA was not synthesized in the Golgi apparatus as are other members of the glycosaminoglycan family. Subsequently, Prehm ((1984) Biochem J. 220:597) showed that, in the presence of added UDP precursors, HA synthesis was ten times greater in disrupted F9 cells as compared to intact cells due to the increased accessibility of the precursor pool. This suggests that the active domain of the HA synthase may be located on the internal surface of the plasma membrane as has been recently hypothesized for the bacterial enzyme (DeAngelis et al. (1993) J. Biol. Chem. 268:19181).

Many attempts have been made to identify the peptide components of both the prokaryotic and eukaryotic HA synthases. In streptococci, Prehm et al.((1986) Biochem. J. 235:887) found proteins of 75, 52, 47, 42 and 34kDa in active HA synthase preparations. Photoaffinity labelling (Van de Rijn et al. (1992) J. Biol. Chem. 267:24302) revealed three proteins of 42, 33

and 27kDa which could bind UDP-GlcA. In eukaryotes, a number of different groups have identified active complexes containing several peptide components ranging in size from 52 to 116kDa (Mian (1986) Biochem J. 237:333 and 343; Ng et al. (1989) J. Biol. Chem. 264:11776; Klewes et al. (1993) Biochem J. 290:791). It can be concluded from the work described above that a complex of plasma membrane-bound proteins is involved in the synthesis and transport of HA in both prokaryotes and eukaryotes. However, none of the above approaches allowed clear identification of the HA synthase. Recent work in streptcocci, involving a tranposon mutagenesis approach, has resulted in identification of the prokaryotic HA synthase as a 42kDa membrane protein. This significant advance should permit an improvement in the understanding of the molecular mechanisms of HA synthesis in streptococci and a subsequent transposition of this knowledge to eukaryotic organisms.

A number of unusual features of HA synthesis have been revealed in the course of studies by various laboratories. If one protein is responsible for HA synthesis, then the synthase must contain independent binding sites for each substrate. Polymerization is rapid, having been estimated using membrane fractions at aproximately 100 sugar units per minute in eukaryotes (Prehm (1983) Biochem J. 211:181). HA synthesis appears to occur by alternate transfer of the growing chain to UDP-GlcA and UDP-NAcGlc. Unusually, chain growth seems to occur at the reducing end (Prehm (1983) Biochem. J. 211:181). Despite numerous attempts, no evidence has been obtained for the involvement of a lipid intermediate in HA synthesis (Sugahara et al (1979) J. Biol. Chem. 254:6252), as is also the case for cellulose synthesis in *Acetobacter xylimon* (Whitfield (1988) Can. J. Microbiol. 34:415).

Previous Attempts at *In Vitro* Synthesis of Hyaluronic Acid

Hyaluronic acid was first synthesized from the activated sugar precursors UDP-GlcNAc and UDP-GlcA using cell extracts (Glaser et al. (1955) Proc. Natl. Acad. Sci. USA 41:253). A soluble and particulate form of the enzyme was found in the vitreous humor (Oesterlin et al. (1968a and 1968b) Exp. Eye Res. 7:497 and 511; Oesterlin (1968) Exp. Eye Res. 7:524; Oesterlin (1969) Exp. Eye Res. 8:27; Jacobson (1978a and 1978b) Exp. Eye Res. 27:247 and 259). The synthase was also characterized in fibroblast fractions (Appel et al. (1979) J. Biol. Chem. 254:12199). A promising approach was the analysis of streptococcal protoplast membranes (Markowitz et al. (1962) Meth. Enzymol. 5:155; Stoolmiller et al. (1969) J. Biol. Chem. 244:236; Sugahara et al. (1979) J.Biol. Chem. 254:6252), which led to a proposed mechanism of HA synthesis (Prehm (1983a) Biochem. J. 211:181; (1983b) Biochem. J. 211:191; (1984) Biochem. J. 220:597). Indeed, it was demonstrated that HA synthase resided in plasma membranes (Philipson et al. (1984) J. Biol. Chem. 259:5017; Prehm (1984) Biochem. J. 220:597).

Several attempts were undertaken to identify and isolate HA synthase. Mian ((1986) Biochem J. 237:333 and 343) tried to dissolve plasma membranes with the detergent NP-40; Ng et al. ((1989) J. Biol. Chem. 264:11176) dissolved plasma membranes with the mild detergent digitonin. In both cases, active fractions were obtained, which contain several proteins. Prehm et al. (1986) and Triscott et al. (1986) tried to isolate the synthase by solubilization of streptococcal membranes with digitonin and cholate. In 1992, the synthase was identified as a 42 kDa protein using photoaffinity-labelled UDP-glucuronic acid (van de Rijn et al. (1992) J. Biol. Chem. 267:24302). After cloning of HA synthase by DeAngelis et al. (1992) and Dougherty

et al. ((1993) J. Exp. Med. 175:1291), antibodies were raised against peptides of the HA synthase and subsequently these antibodies were used to purify the enzyme complex from solubilized streptococcal membranes and from recombinant microorganisms (DeAngelis et al. (1994) Biochem. 33:9033). Purification of the streptococcal HA synthase to homogeneity was achieved by Prehm ((1995) Oral presentation, SFB Meeting, Munster, FRG) by solubilization of membranes with digitonin and phase separation with polyethylenglycol.

JP 02-231093 discloses the synthesis of hyaluronic acid by continuous enzyme reaction using 5'-UMP or its salts.

Preparation of Hyaluronic Acid Oligosaccharides

Up to the present time, HA oligosaccharides have been prepared by digestion of high-molecular weight HA with hyaluronidases and fractionation using standard chromatographic techniques such as those described by West et al. ((1989) Exp. Cell Res. 183:176-196) and EP 88 305255.7. By these known methods, it is only possible to isolate HA oligosaccharides having a molecular weight within the range of 2-4 kDa, i.e., highly polydispersed, and it is not possible to establish the starting and terminating saccharide unit beforehand.

SUMMARY OF THE INVENTION

Recent advances in understanding of the HA biosynthetic apparatus have opened up the possibility of developing a system for *in vitro* enzymatic synthesis of HA. As far as the present inventors are aware, no workers in the field have proposed the possibility of producing HA *in vitro* on an industrial scale. Furthermore, this would be commercially inviable if UDP-

sugars were used as starting materials as they are prohibitively expensive. An innovative feature of the present invention is the use of biochemical recycling reactions to generate UDP-sugars *in situ*, a feature previously undescribed in the field of polysaccharide biosynthesis.

As discussed above, reliable sources of HA exist and are extensively exploited in industry. However, despite the extensive purification carried out on the polymer, concerns are still being expressed about the possibility of contamination with unknown agents such as non-conventional viruses. Additionally, as a consequence of this extensive purification, a polymer of considerable molecular weight polydispersity is obtained. *In vitro* enzymatic synthesis would permit a production of a polymer of extremely high purity and optimized physico-chemical characteristics. The latter properties could be optimized by synthesizing a polymer of the desired molecular weight with a minimized molecular weight polydispersity. Optimization of the technology could also permit synthesis of monodisperse HA oligosaccharides which might demonstrate improved biological activity when compared to the oligosaccharide fractions obtained by hyaluronidase digestion of HA followed by chromatographic separation. In the longer term, the optimization of *in vitro* technology could permit synthesis of novel polymers by modifying the catalytic site of the synthase to obtain enzymes which combine the sugar moieties in varying ways or incorporate alternative sugar components.

The ability to isolate sufficient quantities of highly purified, active HAS allows the development of an efficient synthetic method for the *in vitro* production of HA. HA produced by such a system would offer considerable advantages over the currently available products obtained from animal or bacterial sources.

For example, by using immobilized HAS, it is

possible to produce HA that is free from contaminating proteins or infective agents. By varying the time and/or conditions of the reaction, it is possible to produce a polymer of specific molecular weight, thus facilitating the synthesis of HA of various molecular weights depending upon the intended applications. The ability to regulate the reaction conditions makes it possible to control the polydispersity of the molecular weight, and thus produce a far more homogeneous product than is currently available. Moreover, by employing immobilized HAS, it is possible to produce HA oligosaccharides having defined molecular weights. By incubating the immobilized enzyme alternately with the UDP precursors UDP-glucuronic acid (UDP-GlcA) and UDP-N-acetylglucosamine (UDP-NAcGlc), it is possible to control the molecular weight of the HA oligosaccharides.

It is therefore an object of the present invention to provide a process by which it is possible to obtain hyaluronic acid free from contaminants such as proteins, viruses, and other impurities normally present in this polymer when obtained by conventional extraction methods.

Another object of the present invention is to provide a method for purifying and isolating a protein fraction possessing HAS activity.

Another object of the present invention is to provide a method of immobilizing HAS.

Another object of the present invention is to provide a process for producing HA *in vitro* with different molecular weights, comprising:

incubating a protein or protein mixture active in synthesizing hyaluronic acid in a reaction mixture containing uridine-5'-triphosphate, glucose-1-phosphate, and N-acetylglucosamine-1-phosphate for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetylglucosamine to form hyaluronic acid, and suitable for the formation of UDP-

glucuronic acid from UDP and glucose-1-phosphate and the formation of UDP-N-acetylglucosamine from UDP and N-acetylglucosamine-1-phosphate; and

recovering said hyaluronic acid thus produced.

Another object of the present invention is to provide a process for producing hyaluronic acid *in vitro*, comprising:

incubating a protein or protein mixture active in synthesizing hyaluronic acid in a reaction mixture suitable for the synthesis of hyaluronic acid from UDP-glucose and UDP-N-acetylglucosamine for a time and under conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetyl-glucosamine to form hyaluronic acid, and suitable for the formation of UDP-glucuronic acid from UDP and glucose-1-phosphate and for the formation of UDP-N-acetylglucosamine from UDP and N-acetylglucosamine-1-phosphate,

wherein within said reaction mixture, uridine-5'-triphosphate, glucose-1-phosphate, and N-acetyl-glucosamine-1-phosphate are contained within a single hollowfiber, and

recovering said hyaluronic acid thus produced.

Yet another object of the present invention is to provide a process for producing hyaluronic acid *in vitro*, comprising:

incubating a protein or protein mixture active in synthesizing hyaluronic acid in a reaction mixture suitable for the synthesis of hyaluronic acid from UDP-glucose and UDP-N-acetylglucosamine,

wherein within said reaction mixture, uridine-5'-triphosphate and glucose-1-phosphate are contained within a first hollowfiber, and uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate are contained within a second hollowfiber,

wherein reaction conditions in said first hollowfiber are suitable for the formation of UDP-glucuronic acid from uridine-5'-triphosphate and

glucose-1-phosphate, and wherein reaction conditions in said second hollowfiber are suitable for the formation of UDP-N-acetylglucosamine from uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, and

recovering hyaluronic acid thus produced.

Another object of the present invention is to provide a process for producing hyaluronic acid *in vitro*, comprising:

incubating an immobilized or non-immobilized protein or protein mixture active in synthesizing hyaluronic acid in alternate cycles with a mixture of uridine- 5'-triphosphate and glucose-1-phosphate, and separately with a mixture of uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, in either order,

wherein each alternate cycle is for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetyl-glucosamine to form hyaluronic acid,

wherein each alternate cycle is under reaction conditions suitable for the formation of UDP-glucuronic acid from uridine 5'-triphosphate and glucose-1-phosphate, and for the formation of UDP-N-acetylglucos-amine from uridine 5'-triphosphate and N-acetylglucos-amine-1-phosphate, respectively; and

recovering hyaluronic acid thus produced.

Another object of the present invention is to provide a process for producing hyaluronic acid *in vitro*, comprising:

incubating an immobilized or non-immobilized protein or protein mixture active in synthesizing hyaluronic acid in alternate cycles with a mixture of uridine-5'-triphosphate and glucose-1-phosphate, and separately with a mixture of uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, in either order,

wherein each alternate cycle is for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetyl-

glucosamine to form hyaluronic acid,

wherein said uridine-5'-triphosphate and glucose-1-phosphate are contained within a first hollowfiber under reaction conditions suitable for the formation of UDP-glucuronic acid,

said uridine 5'-triphosphate and N-acetylglucosamine-1-phosphate are contained within a second hollowfiber under reaction conditions suitable for the formation of UDP-N-acetylglucosamine; and

recovering hyaluronic acid thus produced.

A further object of the present invention is to provide hyaluronic acid produced by any of the foregoing processes, wherein the molecular weight of said hyaluronic acid has a polydispersity of between about 1 and about 2.

Another object of the present invention is to provide a pharmaceutical or cosmetic composition containing hyaluronic acid produced by any of the foregoing processes.

Another object of the present invention is the use of hyaluronic acid produced by any of the foregoing processes for the preparation of a pharmaceutical or cosmetic composition.

A further object of the present invention is to provide a process for producing cellulose *in vitro*, comprising:

incubating cellulose synthase in a reaction mixture containing uridine-5'-triphosphate and glucose-1-phosphate for a time and under reaction conditions suitable for the synthesis of cellulose, and suitable for the formation of UDP-glucose from uridine-5'triphosphate and glucose-1-phosphate; and

recovering cellulose thus produced.

Yet a further object of the present invention is to provide a process for producing polymannuronic acid *in vitro*, comprising:

incubating GDP-mannuronic acid polymerase in a

reaction mixture containing guanosine-5'-triphosphate and mannose-1-phosphate for a time and under reaction conditions suitable for the synthesis of polymannuronic acid, and suitable for the formation of GDP-mannuronic acid from guanosine-5'-triphosphate and mannose-1-phosphate, and

recovering polymannuronic acid thus produced.

A still further object of the present invention is to provide a process for producing chitin *in vitro*, comprising:

incubating chitin synthase in a reaction mixture containing uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate for a time and under reaction conditions suitable for the synthesis of chitin, and suitable for the formation of UDP-N-acetylglucosamine from uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, and

recovering chitin thus produced.

Another object of the present invention is to provide a process for producing a polysaccharide *in vitro*, comprising:

incubating the sugar-nucleotide synthase or polymerizing enzyme for said polysaccharide in a reaction mixture containing sugar precursors for said polysaccharide and nucleotide triphosphate carriers for said sugar precursors for a time and under reaction conditions suitable for the synthesis of said polysaccharide, and suitable for the formation of sugar-nucleotide precursors from said nucleotide triphosphate carriers and said sugar precursors, and

recovering said polysaccharide thus produced.

Further scope of the applicability of the present invention will become apparent from the detailed description and drawing provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of

illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features, and advantages of the present invention will be better understood from the following detailed descriptions taken in conjunction with the accompanying drawings, all of which are given by way of illustration only, and are not limitative of the present invention, in which:

Figure 1: Shows the proposed biosynthetic pathway for hyaluronic acid.

Figure 2: Shows separation of an extract of digitonin-solubilized membranes of *S. equisimilis* D181 by anion exchange chromatography. Membranes were loaded with HA by incubating with UDP-GlcA and UDP-NAcGlc. After adding PEG and phase separation, the supernatant is loaded on a DEAE column. (Top) SDS-PAGE analysis of the proteins of fractions 10 - 30. (Bottom) elution profile: hyaluronate synthase activity (boxes) determined according to Prehm ((1983) Biochem. J. 211:181 and 191); NaCl gradient (triangles).

Figure 3: Shows HA produced by immobilized HAS. Immobilized HAS (5 mg of protein/g solid support) was incubated in a 10 ml solution containing 100 mM phosphate buffer, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 1 mM UDP-GlcA and 1 mM UDP-NAcGlc for 2 hours at 37°C. HA was liberated from the immobilized HAS by adding NaCl to a final concentration of 1 M. Immobilized HAS was removed by low centrifugal force (500 x g), and the HA was analysed by SDS-PAGE. Electrophorectic conditions and staining are those described by Moller et al. (1993).

Residual contaminants at the bottom of the gel are probably unincorporated UDP-sugars or very low molecular weight HA.

Figure 4: Shows enzymatic synthesis of hyaluronic acid. The figure demonstrates the incorporation of glucose-1-P in HA using a multienzyme reaction with sugar nucleotide regeneration. The reaction was carried out in 1 ml containing 100 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 4 mM DTT, 50 mM KCl, 10 mM Glc-1-P, 10 mM GlcNAc-1-P, 1 mM UTP, 20 mM PEP, 0.5 mM NAD, 50 LDH, 50 PK, 50 U/ml inorganic pyrophosphatase, 2 U/ml UDP-GlcNAc pyrophosphorylase, 0.5 U UDP-Glc dehydrogenase, 1 U/ml UDP-Glc pyrophosphorylase, and 200 µg HAS for 48 hours at 25°C. The solution was treated with proteinase K and pronase E (see above), and proteins were precipitated by adding TCA to 5% (v/v). The solution was clarified by centrifugation and loaded on a Sepharose CL-4B column, eluted with PBS, and the radioactivity of each fraction was counted. About 90% of the starting amount of Glc-1-P is incorporated in HA.

Figure 5: Shows molecular weight determination by GPC-MALLS of the HA produced *in vitro*. HA was produced as described in Figure 4, using non-radioactively labelled Glc-1-P. After TCA precipitation of the proteins and centrifugation, the supernatant was dialysed (molecular weight cut-off 5000) extensively against water, lyophilized, resuspended in water, and loaded for GPC-MALLS. 1 = HA of molecular weight of about 600,000; 2 = HA of molecular weight of about 540,000; 3 = residual contaminants.

Figure 6: Scheme showing enzymatic synthesis of HA with regeneration of sugar nucleotides.

Figure 7: Scheme showing enzymatic synthesis of HA with regeneration of sugar nucleotides in a hollowfiber enzyme reactor.

Figure 8: Scheme showing enzymatic synthesis of cellulose with regeneration of sugar nucleotides.

Figure 9: Scheme showing enzymatic synthesis of polymannuronic acid with regeneration of sugar nucleotides.

Figure 10: Scheme showing enzymatic synthesis of chitin with regeneration of sugar nucleotides.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description is provided to aid those skilled in the art in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention, as modifications and variations in the embodiments herein discussed may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited herein are herein incorporated by reference in their entirety.

EXAMPLE 1

Isolation and Purification of Hyaluronic Acid Synthase

In order to achieve *in vitro* synthesis of HA and to fully exploit the advantages of the above-described processes, it is necessary to develop a fast and simple method for the purification of active HAS. To purify active HAS, various different approaches can be used, such as phase separation, affinity chromatography, etc.

HAS can be purified according to the following protocol:

1. Streptococcal strains such as *Streptococcus equisimilis* D181 strain obtained from the Rockefeller University strain collection, New York, or other cells capable of producing HAS, such as the species related to Lancefield's A and C groups; eukaryotic cells; or

recombinant organisms which express HAS, including *S. zooepidemicus* strains, such as the strains 68270 and 68222 (Fidia Research Sud, Siracusa, Italy); *S. pyogenes* strains, such as the strains S43/192/4; *S. pyogenes* WF50 or WF51; a *Pasteurella multocida* Carter type A strain 880; recombinant bacterial strains containing a vector or vectors encoding HA synthase, such as the plasmid pPD41, which codes for the streptococcal HA synthase and UDP-Glc dehydrogenase and an undescribed stretch of streptococcal genomic DNA; bacteria containing such plasmids, like *Escherichia coli* and *Enterococcus faecalis* OG1RF; eukaryotic cells, such as fibroblats, chondrocytes, and virally transformed cells (e.g., SV40 transformed 3T3 fibroblasts and RSV transformed chondrocytes), are grown in the desired volume of Todd-Hewitt medium, or any other conventional nutritional medium, at a temperature of between 30°C and 40°C, preferably 37°C, until a final OD_{600nm} of over 0.01 has been reached, preferably about 0.5. One hour before the cells are harvested, a sufficient quantity of hyaluronidase (H3884 from Sigma Chemical Co., St. Louis, MO, USA) is added to the culture to degrade the HA produced and thus facilitate cell harvesting.

2. The cells are pelleted by centrifugation at 6,000 x g for 15 minutes at 4°C, washed once in a suitable buffer, such as a standard cold phosphate buffered saline (PBS), and resuspended in a final volume of between 1 and 100 ml, preferably 20 ml, of cold PBS containing approximately 1mM dithiothreitol (DTT).

3. The suspension is treated by procedures that cause the destruction of the cells, such as sonication at 0°C, and under conditions that ensure that cell destruction is total while minimizing inactivation of the enzyme. Various different sonication conditions can be employed, but the most suitable have proved to be 15 minutes at 120 watts. Unless otherwise stated,

operations should be carried out at 4°C.

Alternatively, cells can be disrupted by French press treatment (e.g., 1000 bar); by treatment with glass beads; or by digestion with cell wall degrading enzymes. One gram of cells in 10 ml standard phosphate buffered saline (PBS) solution or in 50 mM $NaHPO_4/KH_2PO_4$, PH 6.9, 10 mM $MgCl_2$, 5 mM DTT, 30% raffinose, can be digested with lysozyme (1 mg/ml, 37°C, 60 min), mutanolysin (50 μg/ml, 37°C, 60 min), muraminidase (0.1 mg/ml, 37° C, 60 min) or phage lysin (50,000 Units, 37°C, 60 min). After digestion of the cell wall, the protoplasted cells can be lysed by resuspending in hypotonic buffers, such as Tris-malonate or 50 mM $NaHPO_4/KH_2PO_4$, PH 6.9, 10 mM $MgCl_2$, 5 mM DTT. Additionally, to ensure lysis after digestion, sonication, treatment with glass beads, or French press passage can be employed.

4. Cell debris is removed by centrifugation at 10,000 x g for 15 minutes at 4°C, and the supernatant is subjected to ultracentrifugation under conditions that sediment bacterial membranes, preferably 100,000 x g for 30 minutes.

5. The sediment containing the cell membranes is resuspended by mild sonication, for instance 30 seconds at 20 watts, in a small volume of Tris-malonate buffer, 50 mM, pH 7, or any other suitable buffer containing approximately 1 mM DTT, such as PBS. 50 mM $NaHPO_4/KH_2PO_4$, pH 6.9, 150 mM NaCl, 10 mM $MgCl_2$, 5mM DTT, 10% glycerol, can also be used.

The protein concentration is then determined, and the suspension is diluted in the same buffer at a final protein concentration of between about 1 and 10 mg/ml, preferably about 3 mg/ml.

6. To the sample resulting from step 5, a mild

detergent such as digitonin or dodecyl maltoside is added to give a final concentration of between 0.5 and 2%, preferably 1%. The suspension is stirred for about one hour at 0°C and then ultracentrifuged at 100,000 x g for 30 minutes, or under similar conditions, to separate membrane fragments.

7. The synthase can be separated from the detergent in a number of ways.

For example, solubilized membranes can be directly loaded on an ion exchange chromatography column.

Alternatively, HAS can be affinity purified using poly- or monoclonal antibodies against the HA synthase. These antibodies can be immobilized on a solid support.

A preferred method is that described by Parish et al. ((1986) Anal. Biochem. 156:594-602) whereby separation is achieved by adding polyethylene glycol (PEG) 6000. To ensure that the synthase actually separates within the aqueous phase, the protein can be "loaded" with newly-synthesized HA to increase its affinity for the aqueous phase. To this end, the following compounds are added to 5 ml of the supernatant resulting from step 6: between 0.2 and 5 ml of a 50% solution of PEG 6000, preferably 1 ml; 0.05 ml of a solution of 1 M $MgCl_2$, between 0.5 and 5 mg of UDP-N-acetylglucosamine and UDP-glucuronic acid, preferably 2 mg of each. The mixture is then incubated under conditions favouring the synthesis of HA, for example at 37°C for 30 minutes, and is then rapidly cooled to 0°C in an iced salt bath. A further aliquot of 50% PEG 6000 (1 ml) is then added and the solution is vortexed. At this point, the suspension will look turbid, signifying that phase separation has occurred. The suspension is again ultracentrifuged, for example at 100,000 x g for 30 minutes at 4°C.

8. The active HAS can be separated from the

mixture in various ways, including inverse phase chromatography, such as HPLC. The supernatant should preferably be passed through an ion exchange HPLC column, such as a Waters DEAE-protein Pak SWP (7.5 cm x 7.5 mm) column, which has previously been equilibrated with a suitable buffer, such as 50 mM Tris-malonate, pH 7.0, containing a suitable quantity of detergent, such as digitonin, at a final concentration of between 0.01% and 1%, preferably 0.5%. The sample is pumped onto the column at a suitable flow rate, preferably 1 ml/min. Once the supernatant has been loaded, the column is washed with starting buffer, and proteins can be eluted by gradually increasing the salt concentration of the buffer, for example from 0 M to 0.5 M NaCl in 30 minutes, with a constant flow rate of 1 ml/min. Fractions can be gathered at a desired volume, but a volume of 1 ml is advisable.

9. To identify the fractions containing active HAS, a series of analyses are performed on each single fraction. These include determining absorbance at 280 nm, conductivity, and HAS activity by means of radiolabelled precursors, as described by Prehm ((1983) Biochem. J. 211:181-189). In order to associate the activity of the HAS with a given protein or protein fraction, the protein pattern of each fraction can be analysed by polyacrylamide gel electrophoresis, following precipitation of the proteins from a fixed volume of each fraction, for example 200 µl, by a standard technique, such as that described by Wessel and Flügge ((1984) Anal. Biochem. 138:141-143).

The result of a purification experiment is shown in Fig. 2. HAS peak activity resides in the fractions containing a substantially pure protein of approximately 42 kDa, corresponding to the HAS described above.

These results are consistent with those of DeAngelis and Wiegel (1992) and Dougherty and van de

Rijn ((1992) J. Exp. Med. 175:1291), who demonstrated that HAS has a molecular weight of 42 kDa, and further show that the 42 kDa protein constitutes the active synthase, and that this protein is sufficient to permit the *in vitro* synthesis of HA having better characteristics than the HA which is currently being isolated from animal or bacterial sources.

EXAMPLE 2

Immobilization of Isolated and Purified Hyaluronic Acid Synthase

HAS can be immobilized to solid supports (matrices) such as those described by Scouten ((1987) Meth. Enzym. 135:30-65) and used for the *in vitro* synthesis of HA. The following is a typical protocol:

1. In a reaction vessel of the desired size, isolated and purified HAS is diluted in a coupling buffer, such as $NaHCO_3$ (0.1 M, pH 8.3) containing NaCl (0.5 M), or any other suitable buffer such as borate buffer (0.1M, pH 8.3) containing NaCl (0.5M). It is possible to couple 1 to 10 mg, preferably 5 mg, of isolated and purified HAS to 1 g of solid support.

2. The solution resulting from step 1 is mixed with a solid support, such as CNBr-activated Sepharose 4B (Pharmacia), which has previously been washed with 1 mM HCl (200 ml per 1 g of support), or any other suitable kind of support, and incubated under conditions that will allow the HAS to be coupled to the support, for example 16 hours at 4°C using an end-over-end or similar mixer.

3. The support resulting from step 2 is transferred into a solution containing a blocking agent such as 1 M ethanolamine, 0.2 M glycine, or any other

suitable blocking agent, and incubated under conditions that will allow the remaining active groups to be blocked, for instance 2 hours at room temperature.

4. The support resulting from step 3 is washed at room temperature with the coupling buffer, or with any other suitable buffer, for example PBS or 50 mM $NaHPO_4/KH_2PO_4$, PH 6.9, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 10% glycerol, to free it from any excess protein and from the blocking agent.

5. The support resulting from step 4 is stored at 4°C in a storage solution such as PBS, or any other suitable storage solution.

EXAMPLE 3

***In Vitro* Production of Hyaluronic Acid of Varying Molecular Weight Using HAS or Immobilized HAS**

It is possible to synthesize hyaluronic acid of varying molecular weight for various applications using purified HAS or purified and immobilized HAS. Of particular interest are molecular weight fractions in the range of from about 400 to 50,000 Daltons, and those greater than about 2×10^6 Daltons.

As used in the present Example and those that follow, "HAS" refers to pure HAS, a substantially pure protein fraction containing HAS, or a membrane fraction exhibiting HA synthetic activity.

The following is a typical protocol:

1. In a reaction vessel of the desired size, purified HAS or purified and immobilized HAS is diluted in 100 mM HEPES buffer, pH 7.5, or any other suitable buffer, to a final protein concentration of between 0.01 and 1.0 mg/ml, preferably 0.1 mg/ml. Dithriothreitol is

added to the solution to a final concentration of about 1 mM, $MgCl_2$ to a final concentration of about 10 mM, UDP-GlcA and UDP-NAcGlc, both to concentrations of between 0.01 and 5 mM, preferably 1 mM.

2. The mixture is then incubated at a temperature of between 30°C and 40°C, preferably 37°C, for long enough to allow the synthesis of HA, for example, two hours. The mixture is resuspended in a highly saline solution, for example 1 M NaCl, so as to allow the release of HA, and the reaction is stopped by removing immobilized HAS by using low centrifugal forces, for example 500 x g. When the HAS is not immobilized, the reaction is stopped by adding proteinase K and pronase E (from 5 to 100 μg/ml, but preferably 50 μg/ml), and incubating for 30 min at 37° C. The reaction mixture is then subjected to gel filtration on a Sephadex G-25 column, or any other suitable gel permeation column, such as Fractogel HW50, Biogel P-4, Biogel P-6, or Ultrogel AcA202. The HA is eluted with PBS or any other suitable buffer.

3. To determine the molecular weight of the HA produced during steps 1 and 2, radiolabelled precursors such as those described by Prehm ((1983) Biochem. J. 211:181-189) can be used. Alternatively, the HA product can be analysed by SDS-PAGE, as described by Moller et al. ((1993) Anal. Biochem. 209:169-175), by GPC-MALLS (gel permeation chromatography - mid angle laser light scattering), or by any oher method capable of determining the molecular weight of HA.

The molecular weight of the HA synthesized *in vitro* by the use of HAS can be varied by modifying the incubation times and/or reaction conditions. The reaction conditions that can be varied to influence the molecular weight of the final HA product include, but are not limited to, pH, temperature, duration, etc. The

pH can be varied between 4.2 and 8.9; a decrease in the pH produces a decrease in the molecular weight of the HA. The temperature can be varied between 10°C and 40°C; the lower the temperature, the lower the molecular weight of the HA produced. The time range for incubation is between 5 and 120 minutes; shorter incubation times result in HA of lower molecular weight. By way of example, HA having a molecular weight of about 500,000 Daltons requires an incubation time of approximately 30 min at 25°C.

The production of high molecular weight HA by immobilized HAS is shown in Figure 3.

EXAMPLE 4

Production of HA Oligosaccharides and Polysaccharides of Controlled Molecular Weights

It is possible to synthesize HA oligosaccharides and polysaccharides having predefined molecular weights, preferably from about 400 to about 50,000 Daltons, and with given starting and terminating saccharide units, using immobilized HAS and the UDP-precursors UDP-GlcA and UDP-NAcGlc (hereafter designated "A" or "B", respectively). Depending upon the desired initial or terminal saccharide unit, the first (or last) reaction step is started (terminated) with one of the two UDP-precursors A or B. The molecular weight of the hyaluronic acid oligosaccharide or polysaccharide can be controlled by varying the number of times the immobilized HAS is incubated with UDP-GlcA or UDP-NAcGlc.

The following is a typical protocol:

1. In a reaction vessel of the desired size, immobilized HAS is diluted in 100 mM HEPES buffer, pH 7.5, or in any other suitable buffer, to a final concentration of between 0.1 and 10.0 mg/ml, preferably

1.0 mg/ml. To this suspension is added dithiothreitol to a final concentration of about 1 mM and $MgCl_2$ to a final concentration of about 10 mM. Then a UDP-precursor (A or B) is added to the suspension to a final concentration of between 0.01 and 5 mM, preferably 1 mM.

2. The mixture is incubated under suitable conditions, for example at 25°C for 5 minutes.

3. The mixture resulting from step 2 is washed with 100 mM HEPES buffer, pH 7.5, or any other suitable buffer, on a porous glass filter (e.g., with a porosity of G3), and the matrix is recovered.

4. The matrix is resuspended in 100 mM HEPES buffer, pH 7.5, or in any other suitable type of buffer, to a final protein concentration of between 0.1 and 10.0 mg/ml, preferably 1.0 mg/ml. To this suspension are added dithiothreitol to a final concentration of about 1 mM and $MgCl_2$ to a final concentration of about 10 mM. Lastly, the alternate UDP-precursor (A or B) is added to a final concentration of between 0.01 and 5 mM, preferably 1 mM.

5. The subsequent cycles of incubation, washing, matrix recovery, and resuspension are the same as those described in steps 2, 3, and 4, but the UDP-precursors A and B are alternated from cycle to cycle.

6. Once the desired number of cycles has been completed, the mixture is washed with HEPES, or any other suitable buffer, on a porous glass filter (e.g., porosity G3), and the matrix is recovered.

7. The matrix is resuspended and incubated in a highly saline solution, so as to facilitate the release

of the HA oligosaccharide chain from the immobilized HAS, for example in NaCl (1 M) for 30 minutes at 25°C. The matrix is then washed again on a porous glass filter.

8. The filtered product is then subjected to gel filtration on a Sephadex G-25 column or any other suitable gel filtration medium such as Fractogel HW50, Biogel P-4, Biogel P-6, or Ultrogel AcA202. The HA is eluted with PBS or any other suitable buffer.

9. In order to determine the molecular weight of the HA oligosaccharides produced according to steps 1 to 8, radiolabelled precursors can be used, as described by Prehm ((1983) Biochem. J. 211:181-189). Alternatively, the HA thus produced can be analysed by SDS-PAGE, as described by Moller et al. ((1993) Anal. Biochem. 209:169-175), GPC-MALLS, or by any other method capable determining the molecular weight of HA.

EXAMPLE 5

Recycling of UDP and Enzymatic Regeneration of UDP-GlcA and UDP-NAcGlc During the *In Vitro* Production of Hyaluronic Acid

UDP can be recycled, and the UDP-precursors UDP-glucuronic acid (UDP-GlcA) and UDP-N-Acetyl-glucosamine (UDP-NAcGlc) employed in *in vitro* HA synthesis can be enzymatically regenerated, starting from glucose-1-phosphate (Glc-1-P) and N-Acetylglucosamine-1-phosphate (NAcGlc-1-P), phosphoenolpyruvate (PEP), and catalytic quantities of NAD^+ and UDP. Similar methods have been described by Ichikawa et al. ((1992) Anal. Biochem. 202:215-238), Hindsgaul et al. ((1991) Enzymes in Carbohydrate Synthesis, ACS Symposium Series 466:38-50), and Gygax et al. ((1991) Tetrahedron 47:5119-5122).

The following is a typical protocol:

1. In a reaction vessel of the desired volume, purified HAS or purified and immobilized HAS is diluted in a suitable buffer, for example 100 mM HEPES buffer, pH 7.5.

2. To the solution resulting from step 1, the following reagents are added to concentrations of approximately: DTT (between 0.1 and 10 mM, preferably 4 mM), $MgCl_2$ (between 0.1M and 5mM, preferably 10 mM), KCl (between 0.1 M and 100 mM, preferably 50 mM), UTP (between 0.01 and 1 mM, preferably 0,1 mM), NAD^+ and NADH (each between 0.01 and 1 mM, preferably 0.1 mM), Glc-1-P and NAcGlc-1-P (each between 2 mM and 100 mM, preferably 10 mM), PEP (between 2 and 50 mM) pyruvate kinase (PK) and lactate dehydrogenase (LDH) (each between 10 and 1000 U/ml, preferably 50 U/ml), inorganic pyrophosphorylase (PPase) (between 1.2 and 120 U/ml, preferably 50 U/ml), UDP-Glc pyrophosphorylase (between 0.1 and 10 U, preferably 1 U), UDP-GlcNAc pyrophosphorylase (between 0.1 and 20 U, preferably 2 U), and UDP-Glc dehydrogenase (between 0.1 and 10 U/ml, preferably 1 U/ml). The enzymes can also be immobilized on solid supports as described in Example 2, or separated from the HAS by compartmenting them, for example by entrapping them in a hollowfiber system.

3. The solution resulting from step 2 is incubated under suitable reaction conditions, preferably for 48 hours at 25°C, in order to reduce the risk of oxidation of the reaction components, preferably under anaerobic conditions, for example under argon or nitrogen.

The reaction is stopped by removing the enzymes by using low centrifugal forces, for example 500 x g, when they are immobilized, or by removing the compartmenting system, for example by removing the hollowfiber system,

when the enzymes are entrapped within a hollowfiber.

When the enzymes are not immobilized, the reaction is stopped by adding proteinase K and pronase E (from 5 to 100 μg/ml, but preferably 50 μg/ml), and incubating for 30 min at 37°C.

4. The solution resulting from step 3 is chromatographed on a Sephadex G-25 gel permeation column, or any other suitable gel permeation medium such as Fractogel HW50, Biogel P-4, Biogel P-6, or Ultrogel AcA202. The HA is eluted with PBS or any other suitable buffer.

5. To determine the molecular weight of the HA produced according to steps 1, 2 and 3, labelled precursors can be used, such as those described by Prehm ((1983) Biochem. J. 211:181-189). Alternatively, the HA thus produced can be analysed by SDS-PAGE, as described by Moller et al. ((1993) Anal. Biochem. 209:169-175), GPC-MALLS, or by any other method capable determining the molecular weight of HA.

The results of the incorporation of radioactively labeled Glc-1-P in HA are shown in Figure 4.

The results of the molecular weight determination by GPC-MALLS (gel permeation chromatography - mid angle laser light scattering) of the HA produced *in vitro* are shown in Figure 5.

The HA produced in this way has all the necessary characteristics to be used advantageously in pharmaceutical applications, and other related applications, being extremely pure compared to hyaluronic acid purified from conventional sources, and being free from any significant quantities of contaminating proteins, pyrogenic or inflammatory substances, or viruses.

EXAMPLE 6

In Vitro Production of HA Oligosaccharides and Polysaccharides of Controlled Molecular Weights With Recycling of UDP and Enzymatic Regeneration of UDP-GlcA and UDP-NAcGlc

HA oligosaccharides and polysaccharides of predefined molecular weights in the range of from about 400 Daltons to about 50,000 Daltons, with given starting and terminating saccharide units, can be produced in a manner similar to that described in Example 4, taking advantage of the cost-effectiveness of UDP recycling and enzymatic regeneration of UDP-GlcA and UDP-NAcGlc from the relatively inexpensive precursors Glc-1-P and NAcGlc-1-P, as described in Example 5.

Briefly, immobilized or non-immobilized pure HAS, substantially pure HAS, or a membrane fraction exhibiting HA synthetic activity, can be alternately contacted with UDP-GlcA and UDP-NAcGlc regenerating systems under conditions suitable for HA synthesis. In this way, HA oligosaccharides and polysaccharides of defined molecular weights can be cheaply produced.

In another embodiment exemplifying this approach, HAS can be alternately introduced into UDP-GlcA and UDP-NAcGlc regenerating reaction media as described in Example 5, for example by successively recovering immobilized HAS, or compartmentalizing HAS in, for example, a hollowfiber containing HAS in HA synthesis reaction medium.

By any of the foregoing methods, the molecular weight of the HA oligosaccharide or polysaccharide can be controlled by varying the number of times the HAS is incubated with the UDP-GlcA and UDP-NAcGlc regenerating systems.

The following is a typical protocol:

1. In a reaction vessel of the desired volume,

purified HAS, or purified and immobilized HAS, is diluted in 100 mM HEPES buffer, pH 7.5, or in any other suitable buffer, to a final concentration of between 0.1 and 10.0 mg/ml, preferably 1.0 mg/ml. Dithiothreitol is added to the solution to a final concentration of about 1 mM and $MgCl_2$ to a final concentration of about 10 mM.

2. Two hollowfiber systems are employed, one containing the precursors, enzymes, cofactors, etc., required for the regeneration of UDP-GlcA; the other containing the precursors, enzymes, cofactors, etc., required for the regeneration of UDP-NAcGlc.

The buffer system, precursor concentrations, enzyme concentrations, cofactor concentrations, etc., are the same as described in step 2 of Example 5 in the respective hollowfibers.

3. The first hollowfiber containing the regeneration system for either UDP-Glc or UDP-NAcGlc, as desired, is introduced into the reaction vessel, and the mixture is incubated under suitable conditions, for example at 25°C for 5 minutes.

4. The first hollowfiber is removed, and the second hollowfiber containing the alternate UDP-precursor regeneration system is introduced into the reaction vessel and incubated as for the first hollowfiber.

5. Subsequent cycles of hollowfiber introduction and incubation are the same as those described in steps 3 and 4, alternating from cycle to cycle.

6. Once the desired number of cycles has been completed, the matrix is recovered from the reaction vessel by washing on a porous glass filter (e.g., porosity G3), and the matrix is recovered.

7. The matrix is resuspended and incubated in a highly saline solution so as to facilitate the release of the HA oligosaccharide chain from the immobilized HAS, for example in NaCl (1 M) for 30 minutes at 25°C. The matrix is then washed again on a porous glass filter.

8. The filtered product is then subjected to gel filtration. Depending upon the molecular weight of the HA produced, different gel filtration media can be used. For HA oligosaccharides with lower molecular weights, a suitable gel filtration medium is Sephadex G-10 or any other similar gel filtration medium such as Biogel P-2 or Sephadex G-15. For larger oligo- or polysaccharides up to 50,000 Daltons, a suitable gel filtration medium is Fractogel HW40 or any other similar gel filtration medium such as Fractogel HW50. The HA is eluted with PBS or any other suitable buffer.

9. When the HAS is not immobilized, the hollow fiber is removed, and the reaction is stopped by adding proteinase K and pronase E (from 5 to 100 μg/ml, but preferably 50 μg/ml) and incubating for 30 min at 37° C. The reaction mixture is then subjected to gel filtration. Depending upon the molecular weight of the HA produced, different gel filtration media can be used. For HA oligosaccharides with lower molecular weights, a suitable gel filtration medium is Sephadex G-10 or any other similar gel filtration medium such as Biogel P-2 or Sephadex G-15. For larger oligo- or polysaccharides up to 50,000 Daltons, a suitable gel filtration medium is Fractogel HW40 or any other similar gel filtration medium such as Fractogel HW50. The HA is eluted with PBS or any other suitable buffer.

10. In order to determine the molecular weight of the HA oligosaccharides and polysaccharides produced

according to steps 1 to 9, radiolabelled precursors can be used, as described by Prehm ((1983) Biochem. J. 211:181-189). Alternatively, the HA thus produced can be analysed by SDS-PAGE, as described by Moller et al. ((1993) Anal. Biochem. 209:169-175), GPC-MALLS, or by any other method capable determining the molecular weight of HA.

EXAMPLE 7

Compartmentalized System With Recycling of UDP and Enzymatic Regeneration of UDP-GlcA and UDP-NAcGlc For the *In Vitro* Synthesis of Hyaluronic Acid

HA can also be synthesized using a system wherein UDP is recycled and UDP-GlcA and UDP-NAcGlc are enzymatically regenerated during the synthesis of HA *in vitro* by employing a "two pot system" wherein hollowfibers are employed to compartmentalize the recycling and regeneration reactions.

A typical reaction protocol is as follows.

1. Two hollowfiber systems are submerged in a reaction solution containing HAS, DTT, and $MgCl_2$, as described in Example 3. One hollowfiber contains the precursors, enzymes, cofactors, etc. required for the regeneration of UDP-GlcA; the other hollowfiber contains the precursors, enzymes, cofactors, etc. required for the regeneration of UDP-NAcGlc. This is schematically shown in Figure 6.

Alternatively, the enzymes required for the regeneration of the UDP-precursors can be contained in a single hollowfiber system which is submerged in an HAS-containing reaction solution.

In either case, the buffer system, precursor concentrations, enzyme concentrations, cofactor concentrations, etc., are the same as described in step 2 of Example 5.

2. The reaction solution is incubated under suitable reaction conditions, preferably for 48 hours at 25°C, in order to reduce the risk of oxidation of the reaction components, preferably under anaerobic conditions, for example under argon or nitrogen.

3. The reaction is stopped by removing the enzymes entrapped within the hollowfiber.

4. The reaction solution containing HAS is treated with proteinase K and pronase E (from 5 to 100 μg/ml, preferably 50 μg/ml), and incubated for 30 min at 37°C. If the HAS is immobilized, the enzyme is removed by using low centrifugal forces, for example 500 x g.

5. The solution resulting from step 4 is chromatographed on a Sephadex G-25 gel permeation column, or any other suitable gel permeation medium such as Fractogel HW50, Biogel P-4, Biogel P-6, or Ultrogel AcA202. The HA is eluted with PBS or any other suitable buffer.

6. To determine the molecular weight of the HA produced according to steps 1-5, labelled precursors can be used, such as those described by Prehm ((1983) Biochem. J. 211:181-189).. Alternatively, the HA thus produced can be analysed by SDS-PAGE, as described by Moller et al. ((1993) Anal. Biochem. 209:169-175), GPC-MALLS, or by any other method capable determining the molecular weight of HA.

EXAMPLE 8

In Vitro Synthesis of Cellulose

Cellulose can be synthesized *in vitro* using the sugar precursor UDP-glucose (UDP-Glc) and cellulose synthase, which can be isolated from *Acetobacter xylinum*

or protoplasted plant cells (Wong et al. (1990) Proc. Natl Acad. Sci. USA 87:8130-8134).

As shown in Figure 8, the reaction system includes cellulose synthase, and the regeneration system for UDP-Glc includes glucose-1-P, phosphoenolpyruvate, $MgCl_2$, DTT, pyruvate kinase, UDP-Glc pyrophosphorylase, and inorganic pyrophosphatase. Reaction conditions are similar to those described in Example 5.

EXAMPLE 9

***In Vitro* Synthesis of Polymannuronic Acid**

Polymannuronic acid (PM) is an immunostimulating agent, and much research has been done to manipulate the fermentation conditions of alginate-producing bacterial strains such as *Pseudomonas aeruginosa*, *P. fluoreszens*, and *Azotobacter vinelandi*. PM can be synthesized *in vitro* using GDP-mannuronic acid polymerase (Gacesa et al. (1990) Psedomonas Infection and Alginates, Biochemistry, Genetics and Pathology, Chapman and Hall, London).

As shown in Figure 9, the reaction system includes GDP-mannuronic acid polymerase, and the regeneration system for GDP-mannuronic acid contains mannose-1-P, phosphoenolpyruvate, $MgCl_2$, DTT, pyruvate kinase, GDP-mannose pyrophosphorylase, GDP-mannose dehydrogenase, and inorganic pyrophosphatase. Reaction condition are similar to those described in Example 5.

EXAMPLE 10

***In Vitro* Synthesis of Chitin**

As shown in Figure 10, chitin can be synthesized *in vitro* using the sugar precursor UDP-N-Acetylglucos-amine (UDP-NAcGlcA) and chitin synthase, which can be isolated from *Saccharomyces cerevisiae*. Alternatively, a cloned enzyme from *S. cerevisiae* can be used (Silverman (1989)

Yeast 5:459-467; Bulawa (1992) Mol. Cell Biol. 12:1764-1776; Cabib et al. (1983) Proc. Natl. Acad. Sci. USA 80:3318-3321).

The regeneration system for UDP-NAcGlcA includes N-acetylglucosamine-1-P, phosphoenolpyruvate, $MgCl_2$, DTT, pyruvate kinase, UDP-NAcGlc pyrophosphorylase, and inorganic pyrophosphatase. Reaction conditions are similar to those described in Example 5.

EXAMPLE 11

PHARMACEUTICAL COMPOSITIONS

Pharmaceutical compositions containing an effective amount of hyaluronic acid prepared according to the methods of the present invention, alone or in association with one or more active principles, pharmacologically acceptable carriers, diluents, or excipients, can be used advantageously in the medical/pharmaceutical field, for example in ophthalmology, in the area of tissue repair, and in rheumatology.

These pharmaceutical compositions can be administered by the topical, intra-articular, ophthalmic, systemic, and peritoneal routes. Such pharmaceutical compositions have been described in European Patent 0 138 572 B1, and in PCT publication WO 92/18543.

These compositions typically contain hyaluronic acid in an amount of from about 0.1 mg/ml to about 100 mg/ml.

For topical administration, these compositions can be in the form of creams, gels, ointments, bandages, and gauzes.

For ophthalmic use, they can be in the form of eye drops, viscoelastic surgical aids, lenses, etc.

For systemic administration, pharmaceutical preparations for intravenous or intramuscular

adminstration are suitable, such as ampoules or vials.

Pharmaceutical compositions containing HA having a molecular weight of from about 400 Daltons to about 50,000 Daltons are useful in the area of tissue repair, where promotion of angiogenesis is required. Pharmaceutical compositions containing HA having a molecular weight between about 2×10^6 and about 3×10^6 Daltons can be administered to human or animal subjects to inhibit the formation of hypertrophic scars and keloids arising at wound sites resulting from injury or surgical intervention.

In addition, pharmaceutical compositions containing HA having a molecular weight greater than about 500,000 Daltons can be administered intraarticularly for the treatment of arthritis.

Furthermore, formulations based on HA having a molecular weight greater than about 750,000 Daltons can be used in ophthamology.

The above-described pharmaceutical compositions can be applied topically to the wound site in the form of a liquid, cream, gel, ointment, spray, wound dressing, medicated biomaterial such as a film, gauze, threads, etc., or in the form of an intradermal injection at the time of wound formation or suturing.

Doses of high molecular weight HA depend on the individual need of the patient, on the desired effect, and on the route of administration, and can typically be in the range of from about 0.1 mg to about 100 mg per inch or square inch of incision or wound, respectively.

The administration of these pharmaceutical compositions to the wound site can be continued on a daily basis as required until such time as the healing process has been completed in order to avoid scar or keloid formation.

The invention being thus described, it is obvious that the same can be modified in various ways. Such modifications are not to be regarded as a departure from

the spirit and scope of the invention, and all such modifications that would appear obvious to one skilled in the art are intended to be included within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A process for producing hyaluronic acid *in vitro*, comprising:

incubating a protein or protein mixture active in synthesizing hyaluronic acid in a reaction mixture containing uridine-5'-triphosphate, glucose-1-phosphate, and N-acetylglucosamine-1-phosphate for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetylglucosamine to form hyaluronic acid, and suitable for the formation of UDP-glucuronic acid from UDP and glucose-1-phosphate and the formation of UDP-N-acetylglucosamine from UDP and N-acetylglucosamine-1-phosphate; and

recovering said hyaluronic acid thus produced.

2. The process of claim 1, wherein said protein or said protein mixture is immobilized on a solid support.

3. The process of claim 1, wherein the molecular weight of said hyaluronic acid thus produced can be varied by varying said time and said reaction conditions.

4. The process of claim 1, wherein the molecular weight of said hyaluronic acid is in the range of from about 400 Daltons to about 50,000 Daltons, or from about 50,000 Daltons to about 10,000,000 Daltons.

5. A process for producing hyaluronic acid *in vitro*, comprising:

incubating a protein or protein mixture active in synthesizing hyaluronic acid in a reaction mixture suitable for the synthesis of hyaluronic acid from UDP-glucose and UDP-N-acetylglucosamine for a time and under conditions suitable for the polymerization of UDP-

glucuronic acid and UDP-N-acetyl-glucosamine to form hyaluronic acid, and suitable for the formation of UDP-glucuronic acid from UDP and glucose-1-phosphate and for the formation of UDP-N-acetylglucosamine from UDP and N-acetylglucosamine-1-phosphate,

wherein within said reaction mixture, uridine-5'-triphosphate, glucose-1-phosphate, and N-acetyl-glucosamine-1-phosphate are contained within a single hollowfiber, and

recovering said hyaluronic acid thus produced.

6. A process for producing hyaluronic acid *in vitro*, comprising:

incubating a protein or protein mixture active in synthesizing hyaluronic acid in a reaction mixture suitable for the synthesis of hyaluronic acid from UDP-glucose and UDP-N-acetylglucosamine,

wherein within said reaction mixture, uridine-5'-triphosphate and glucose-1-phosphate are contained within a first hollowfiber, and uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate are contained within a second hollowfiber,

wherein reaction conditions in said first hollowfiber are suitable for the formation of UDP-glucuronic acid from uridine-5'-triphosphate and glucose-1-phosphate, and wherein reaction conditions in said second hollowfiber are suitable for the formation of UDP-N-acetylglucosamine from uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, and

recovering hyaluronic acid thus produced.

7. A process for producing hyaluronic acid *in vitro*, comprising:

incubating an immobilized or non-immobilized protein or protein mixture active in synthesizing hyaluronic acid in alternate cycles with a mixture of uridine- 5'-triphosphate and glucose-1-phosphate, and

separately with a mixture of uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, in either order,

wherein each alternate cycle is for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetylglucosamine to form hyaluronic acid,

wherein each alternate cycle is under reaction conditions suitable for the formation of UDP-glucuronic acid from uridine 5'-triphosphate and glucose-1-phosphate, and for the formation of UDP-N-acetylglucosamine from uridine 5'-triphosphate and N-acetylglucosamine-1-phosphate, respectively; and

recovering hyaluronic acid thus produced.

8. A process for producing hyaluronic acid *in vitro*, comprising:

incubating an immobilized or non-immobilized protein or protein mixture active in synthesizing hyaluronic acid in alternate cycles with a mixture of uridine-5'-triphosphate and glucose-1-phosphate, and separately with a mixture of uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, in either order,

wherein each alternate cycle is for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetylglucosamine to form hyaluronic acid,

wherein said uridine-5'-triphosphate and glucose-1-phosphate are contained within a first hollowfiber under reaction conditions suitable for the formation of UDP-glucuronic acid,

said uridine 5'-triphosphate and N-acetylglucosamine-1-phosphate are contained within a second hollowfiber under reaction conditions suitable for the formation of UDP-N-acetylglucosamine; and

recovering hyaluronic acid thus produced.

9. The process of any one of claims 7 or 8, wherein the molecular weight of said hyaluronic acid thus produced is varied by varying the number of said alternate cycles.

10. The process of any one of claims 7 or 8, wherein said hyaluronic acid thus produced has a molecular weight in the range of from about 400 Daltons to about 50,000 Daltons.

11. The process of any one of claims 1 to 10, wherein said protein or protein mixture active in synthesizing hyaluronic acid is isolated from a naturally occurring or transformed eukaryotic or prokaryotic cell.

12. The process of claim 11, wherein said protein or protein mixture active in synthesizing hyaluronic acid contains hyaluronic acid synthase or a variant form thereof that is active in synthesizing hyaluronic acid.

13. Hyaluronic acid produced by the process of any one of claims 1 to 12, wherein the molecular weight of said hyaluronic acid has a polydispersity of between about 1 and about 2.

14. Hyaluronic acid produced by the process of any one of claims 8 to 10, wherein the molecular weight of said hyaluronic acid has a polydispersity of about 1.

15. Hyaluronic acid produced by the process of any one of claims 1 to 12, wherein said hyaluronic acid is free of any significant quantities of contaminating viruses, proteins, pyrogenic, or inflammatory substances.

16. A pharmaceutical or cosmetic composition

containing hyaluronic acid produced by the process of any one of claims 1-12.

17. Use of hyaluronic acid produced by the process of any one of claims 1-12 for the preparation of a pharmaceutical or cosmetic composition.

18. Use of said pharmaceutical composition of claim 17 in ophthalmology, tissue repair, or rheumatology.

19. The use of claim 18, wherein said pharmaceutical composition is administered topically, intra-articularly, or systemically.

20. A biomaterial containing hyaluronic acid produced by the process of any one of claims 1-12.

21. Use of hyaluronic acid produced by the process of any one of claims 1-12 for the preparation of a biomaterial.

22. The use of claim 10, wherein said biomaterial is selected from the group consisting of a thread, a film, a membrane, a gauze, a sponge, a microsphere, a capsule, a microcapsule, and a device for controlled release of a biologically or pharmaceutically active substance.

23. A process for producing cellulose *in vitro*, comprising:

incubating cellulose synthase in a reaction mixture containing uridine-5'-triphosphate and glucose-1-phosphate for a time and under reaction conditions suitable for the synthesis of cellulose, and suitable for the formation of UDP-glucose from uridine-5'triphosphate and glucose-1-phosphate; and

recovering cellulose thus produced.

24. A process for producing polymannuronic acid *in vitro*, comprising:

incubating GDP-mannuronic acid polymerase in a reaction mixture containing guanosine-5'-triphosphate and mannose-1-phosphate for a time and under reaction conditions suitable for the synthesis of polymannuronic acid, and suitable for the formation of GDP-mannuronic acid from guanosine-5'-triphosphate and mannose-1-phosphate, and

recovering polymannuronic acid thus produced.

25. A process for producing chitin *in vitro*, comprising:

incubating chitin synthase in a reaction mixture containing uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate for a time and under reaction conditions suitable for the synthesis of chitin, and suitable for the formation of UDP-N-acetylglucosamine from uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, and

recovering chitin thus produced.

26. A process for producing a polysaccharide *in vitro*, comprising:

incubating the sugar-nucleotide synthase or polymerizing enzyme for said polysaccharide in a reaction mixture containing sugar precursors for said polysaccharide and nucleotide triphosphate carriers for said sugar precursors for a time and under reaction conditions suitable for the synthesis of said polysaccharide, and suitable for the formation of sugar-nucleotide precursors from said nucleotide triphosphate carriers and said sugar precursors, and

recovering said polysaccharide thus produced.

27. A polysaccharide produced by the process of any one of claims 23-26.

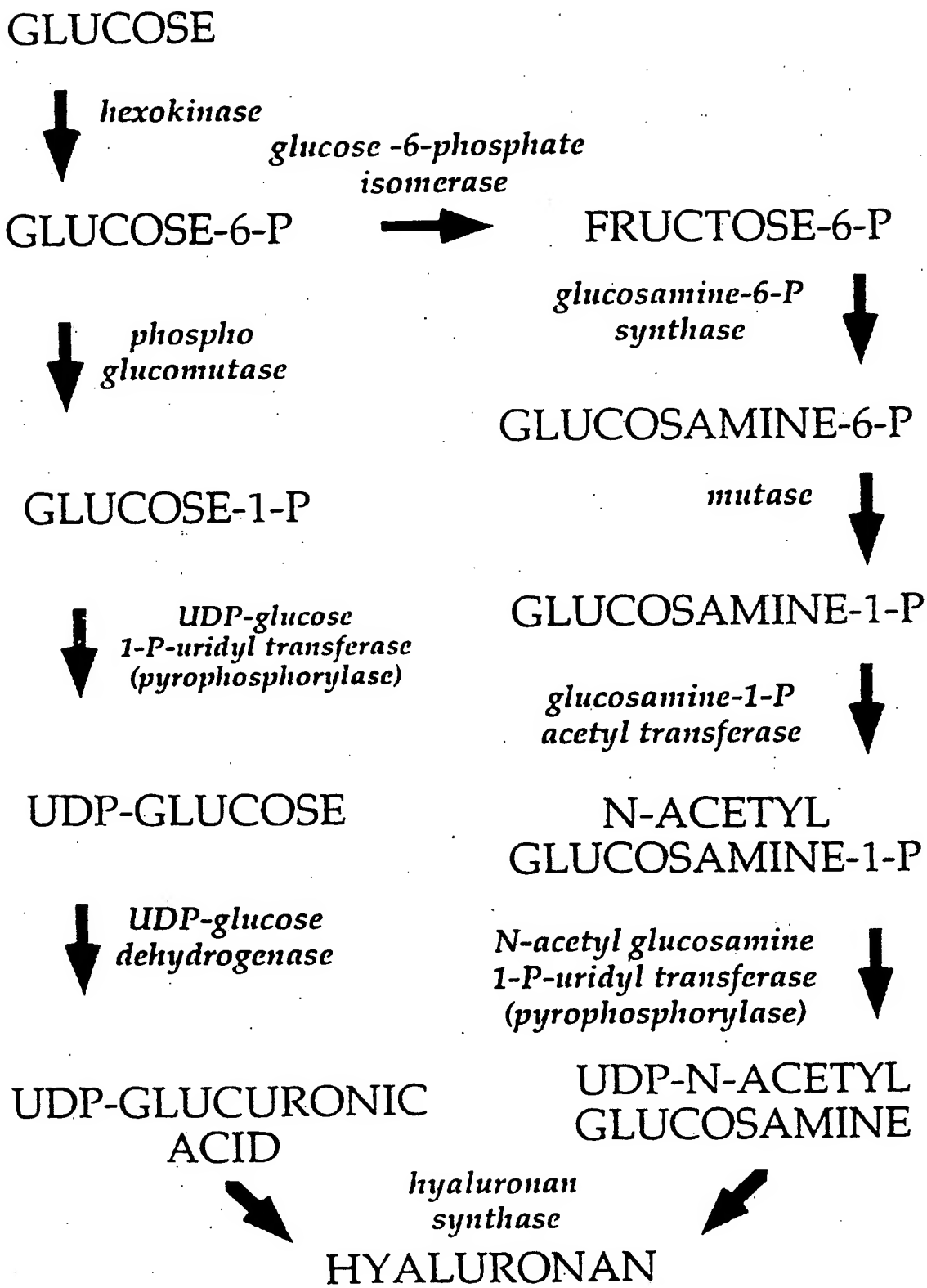

GLUCOSE
hexokinase
glucose -6-phosphate isomerase
GLUCOSE-6-P
FRUCTOSE-6-P
glucosamine-6-P synthase
phospho glucomutase
GLUCOSAMINE-6-P
GLUCOSE-1-P
mutase
GLUCOSAMINE-1-P
UDP-glucose 1-P-uridyl transferase (pyrophosphorylase)
glucosamine-1-P acetyl transferase
UDP-GLUCOSE
N-ACETYL GLUCOSAMINE-1-P
UDP-glucose dehydrogenase
N-acetyl glucosamine 1-P-uridyl transferase (pyrophosphorylase)
UDP-GLUCURONIC ACID
UDP-N-ACETYL GLUCOSAMINE
hyaluronan synthase
HYALURONAN

Fig. 1

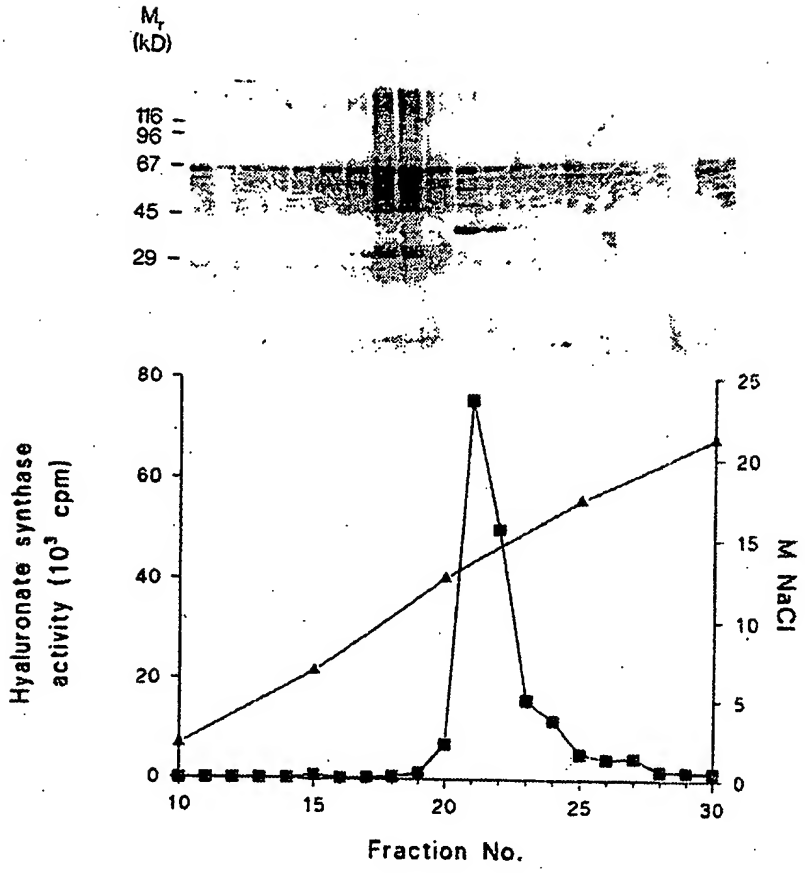

M_r (kD)
116
96
67
45
29
Hyaluronate synthase activity (10^3 cpm)
80
60
40
20
0
25
20
15
10
5
0
M NaCl
10
15
20
25
30
Fraction No.

Fig. 2

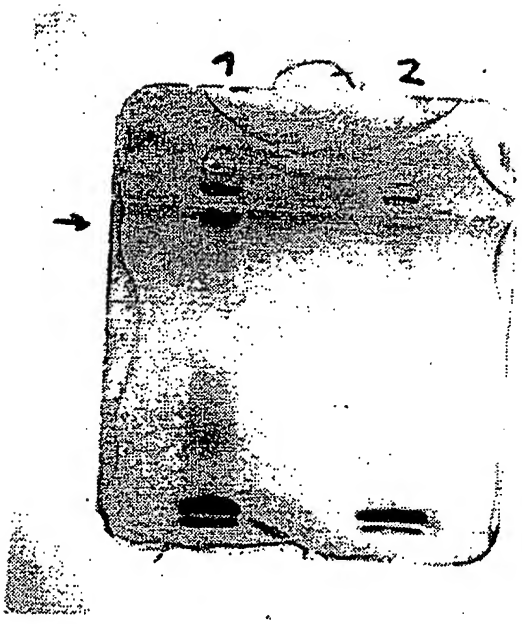


Fig. 3

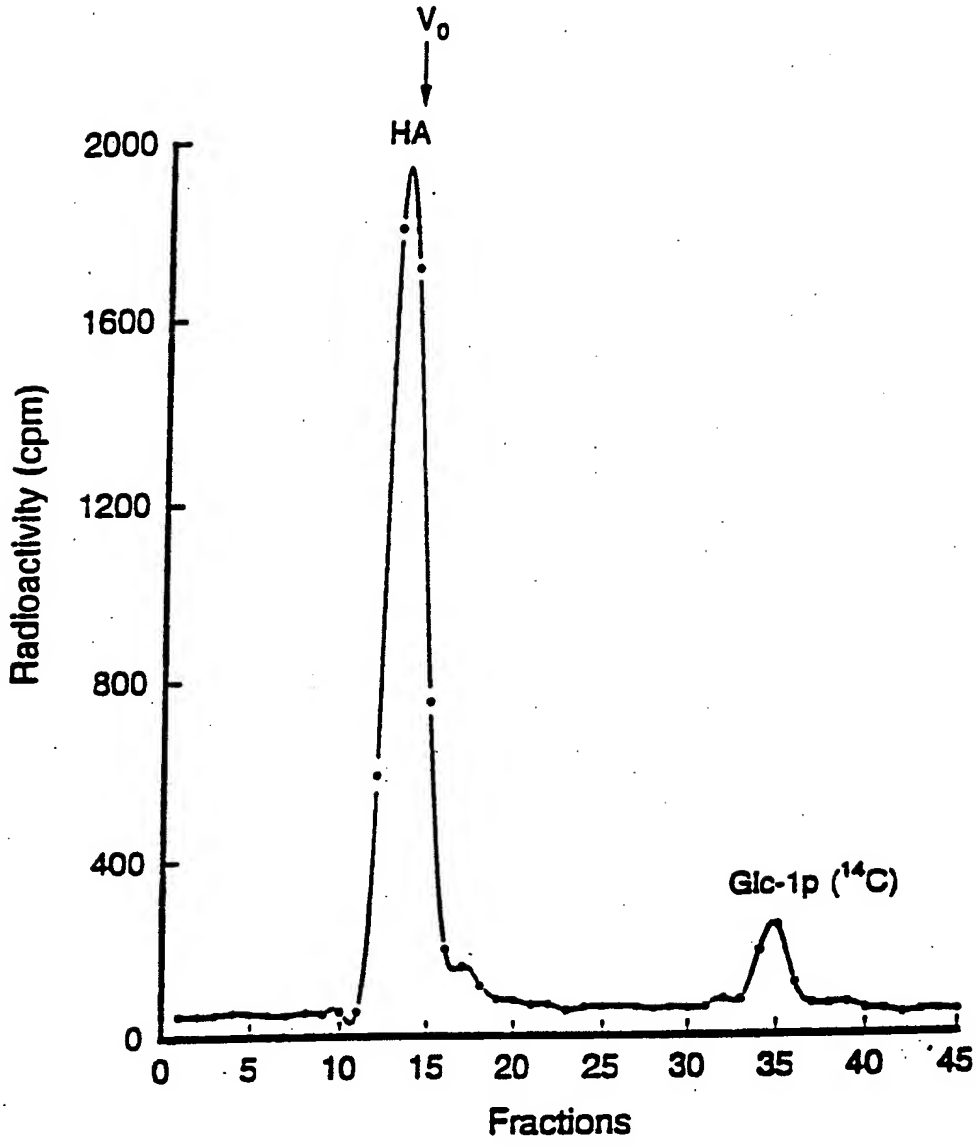

V_0
HA
2000
1600
1200
800
400
0
Radioactivity (cpm)
Glc-1p (^{14}C)
0
5
10
15
20
25
30
35
40
45
Fractions

Fig. 4

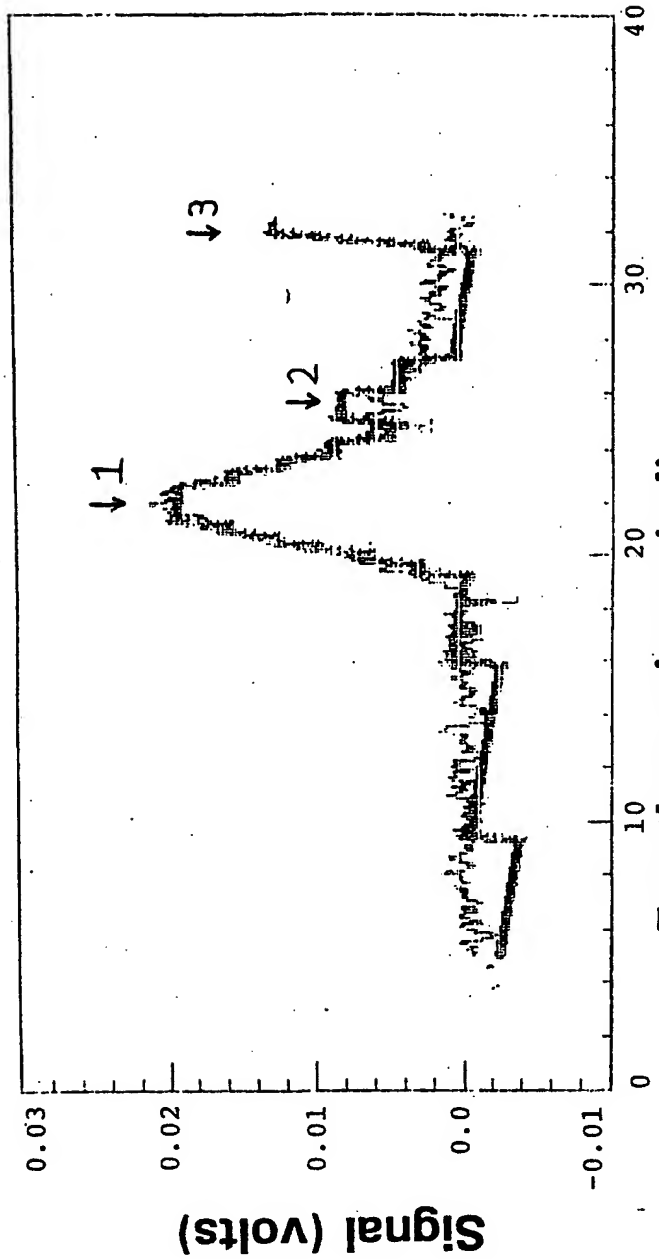

Signal (volts)
0.03
0.02
0.01
0.0
-0.01
Fraction volume (ml)
0
10
20
30
40
1
2
3

Fig. 5

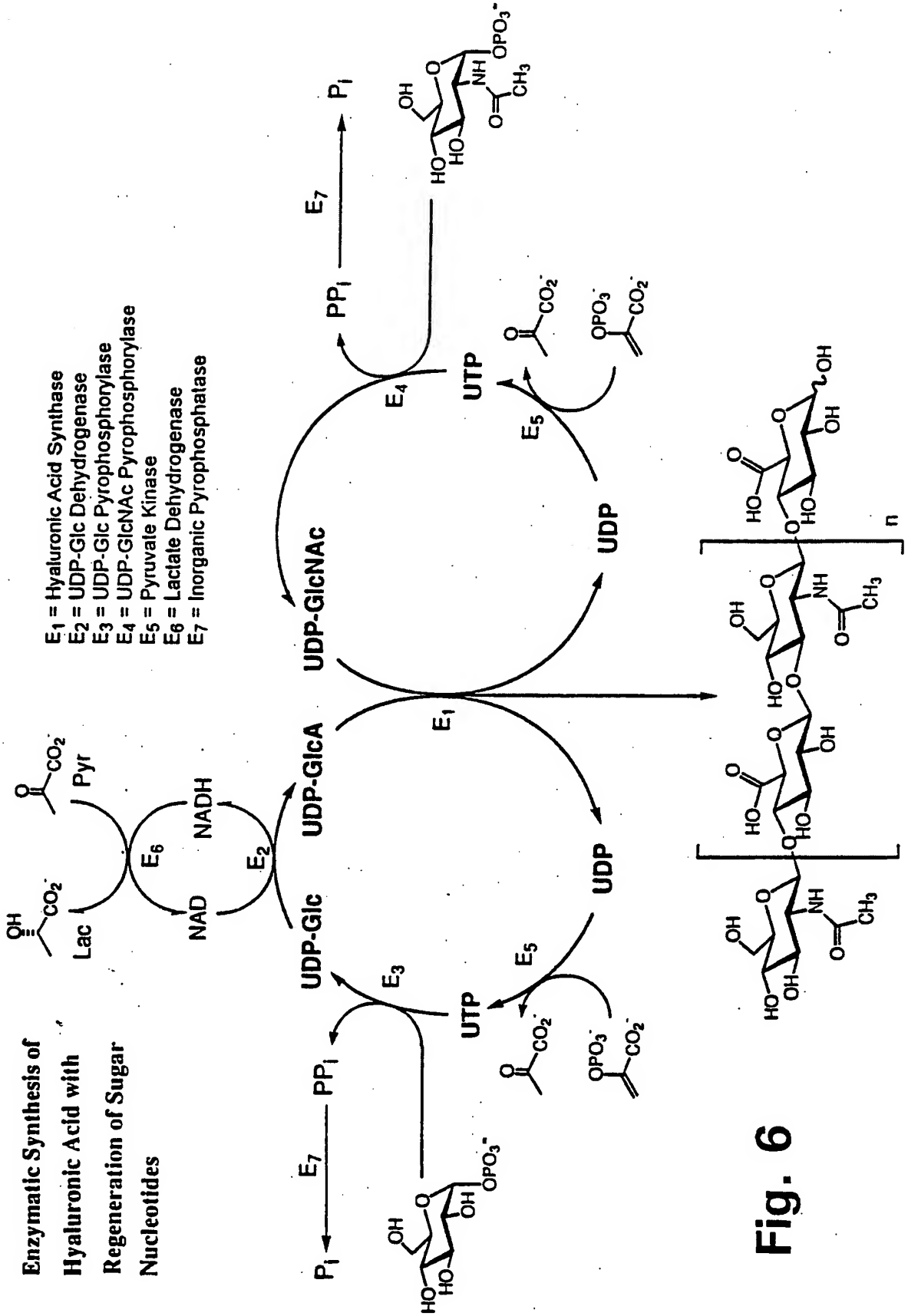

Enzymatic Synthesis of
Hyaluronic Acid with
Regeneration of Sugar
Nucleotides
E1 = Hyaluronic Acid Synthase
E2 = UDP-Glc Dehydrogenase
E3 = UDP-Glc Pyrophosphorylase
E4 = UDP-GlcNAc Pyrophosphorylase
E5 = Pyruvate Kinase
E6 = Lactate Dehydrogenase
E7 = Inorganic Pyrophosphatase
Lac
Pyr
NAD
NADH
UDP-Glc
UDP-GlcA
UDP-GlcNAc
UTP
UDP
PPi
Pi
n

Fig. 6

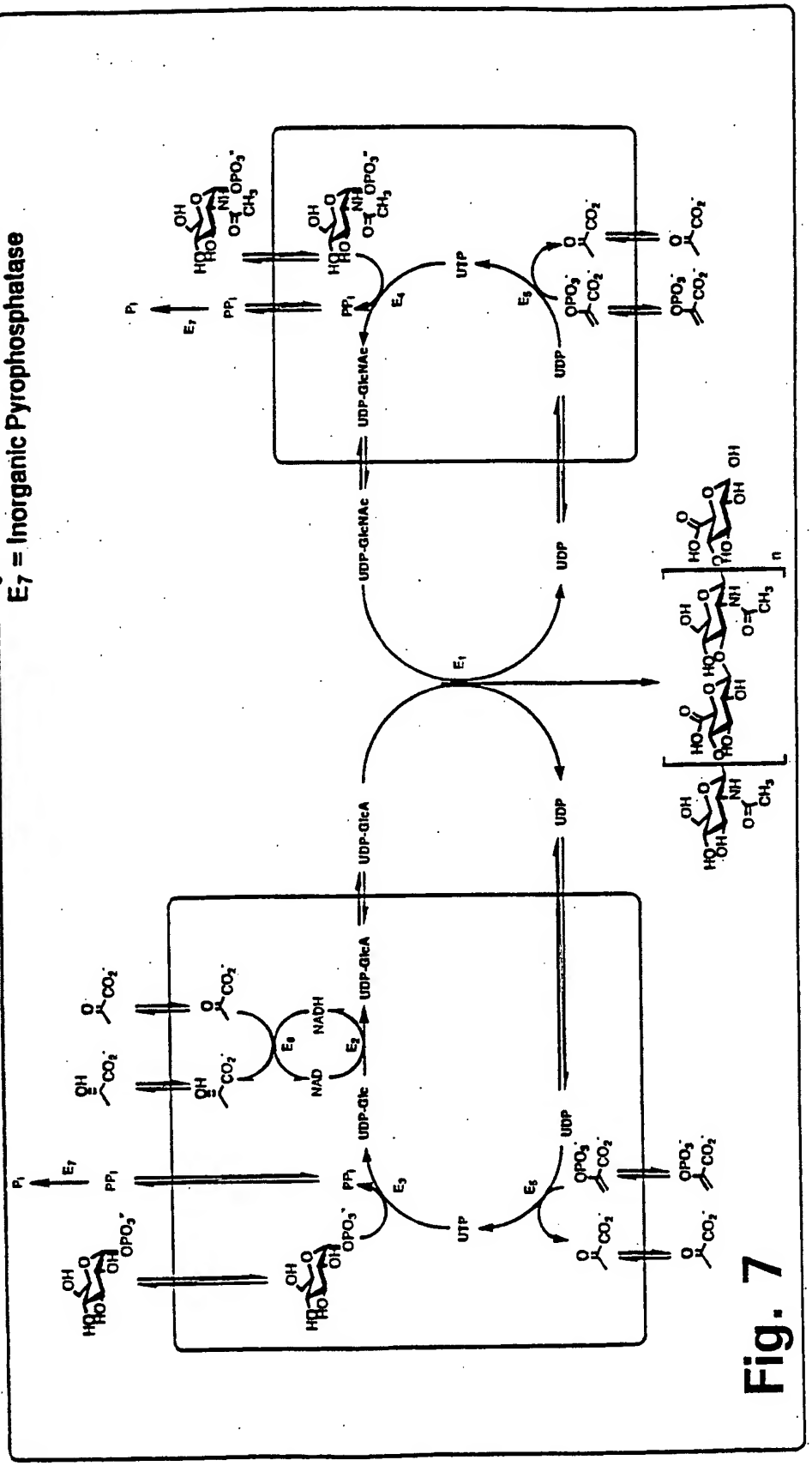

Hollow Fiber Enzyme Reactor
for the
Synthesis of Hyaluronic Acid
with
Regeneration of Sugar Nucleotides
E1 = Hyaluronic Acid Synthase
E2 = UDP-Glc Dehydrogenase
E3 = UDP-Glc Pyrophosphorylase
E4 = UDP-GlcNAc Pyrophosphorylase
E5 = Pyruvate Kinase
E6 = Lactate Dehydrogenase
E7 = Inorganic Pyrophosphatase
UDP-GlcA
UDP-Glc
UDP-GlcNAc
UDP
UTP
NADH
NAD
Pi
PPi

Fig. 7

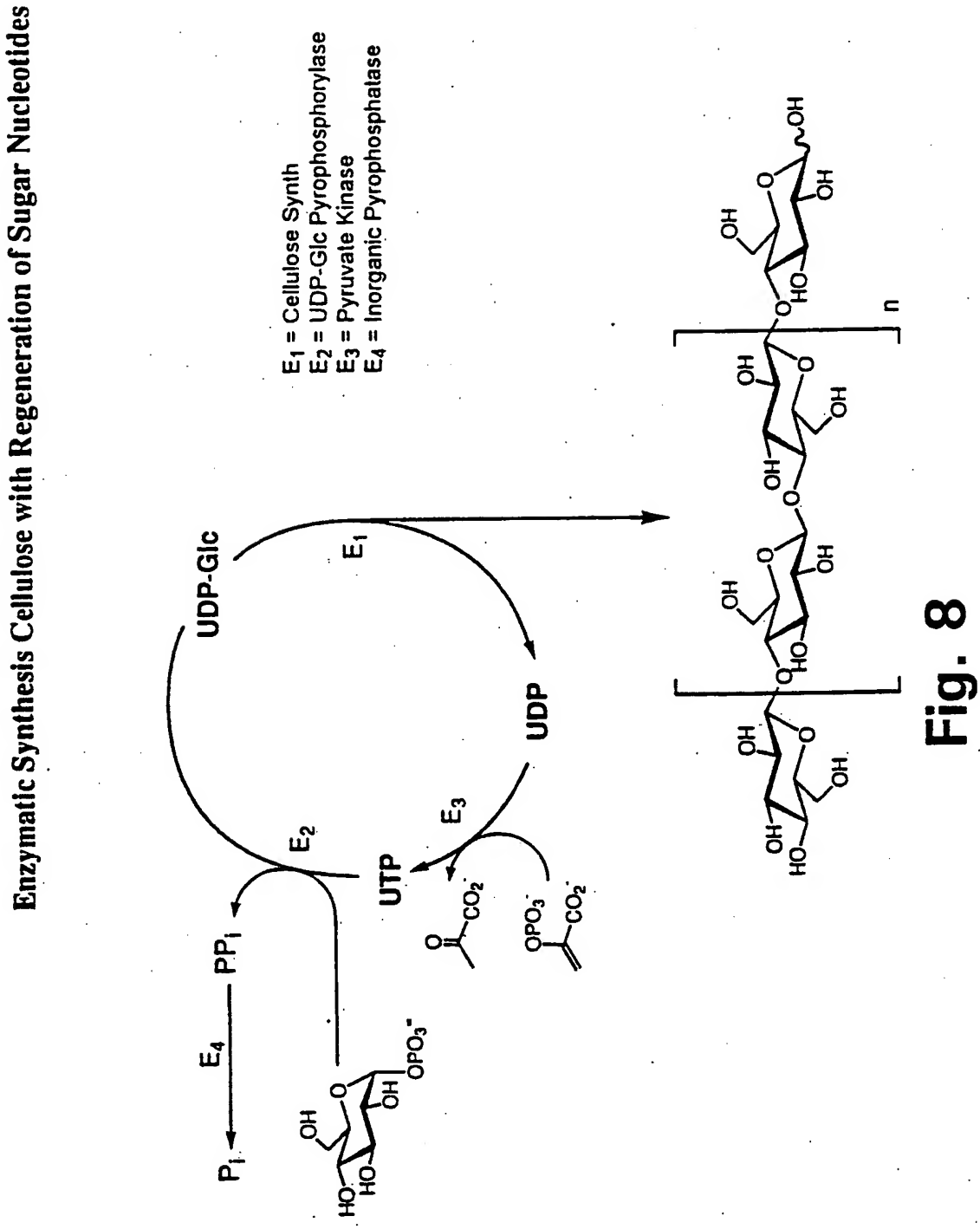

Enzymatic Synthesis Cellulose with Regeneration of Sugar Nucleotides
E1 = Cellulose Synth
E2 = UDP-Glc Pyrophosphorylase
E3 = Pyruvate Kinase
E4 = Inorganic Pyrophosphatase
UDP-Glc
E1
UDP
E3
E2
UTP
E4
PPi
Pi
n

Fig. 8

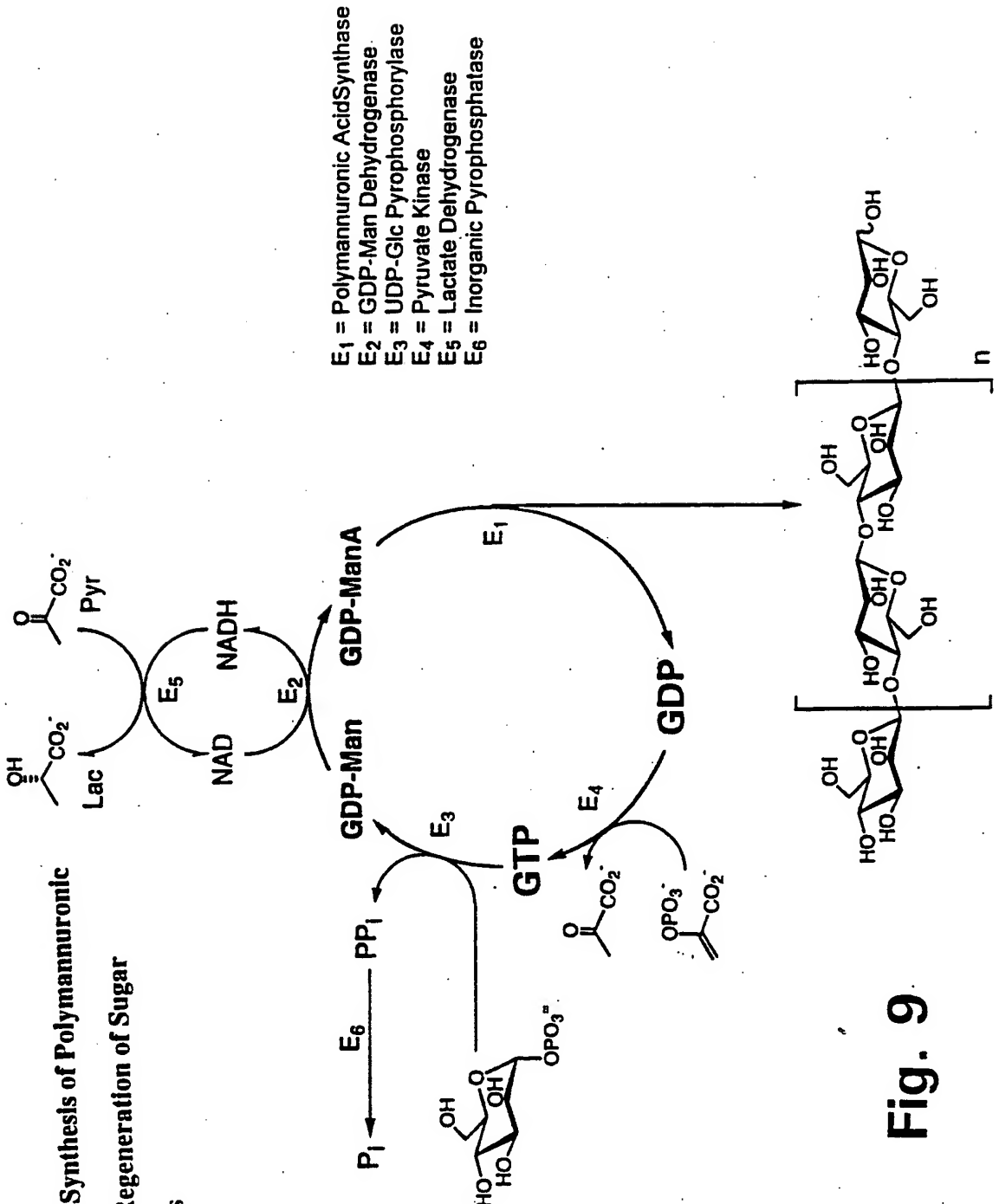

E1 = Polymannuronic AcidSynthase
E2 = GDP-Man Dehydrogenase
E3 = UDP-Glc Pyrophosphorylase
E4 = Pyruvate Kinase
E5 = Lactate Dehydrogenase
E6 = Inorganic Pyrophosphatase
Enzymatic Synthesis of Polymannuronic Acid with Regeneration of Sugar Nucleotides
Pyr
Lac
NADH
NAD
E5
E2
GDP-ManA
GDP-Man
E1
GDP
E3
GTP
E4
E6
PPi
Pi
n

Fig. 9

Enzymatic Synthesis of Chitin with Regeneration of Sugar Nucleotides

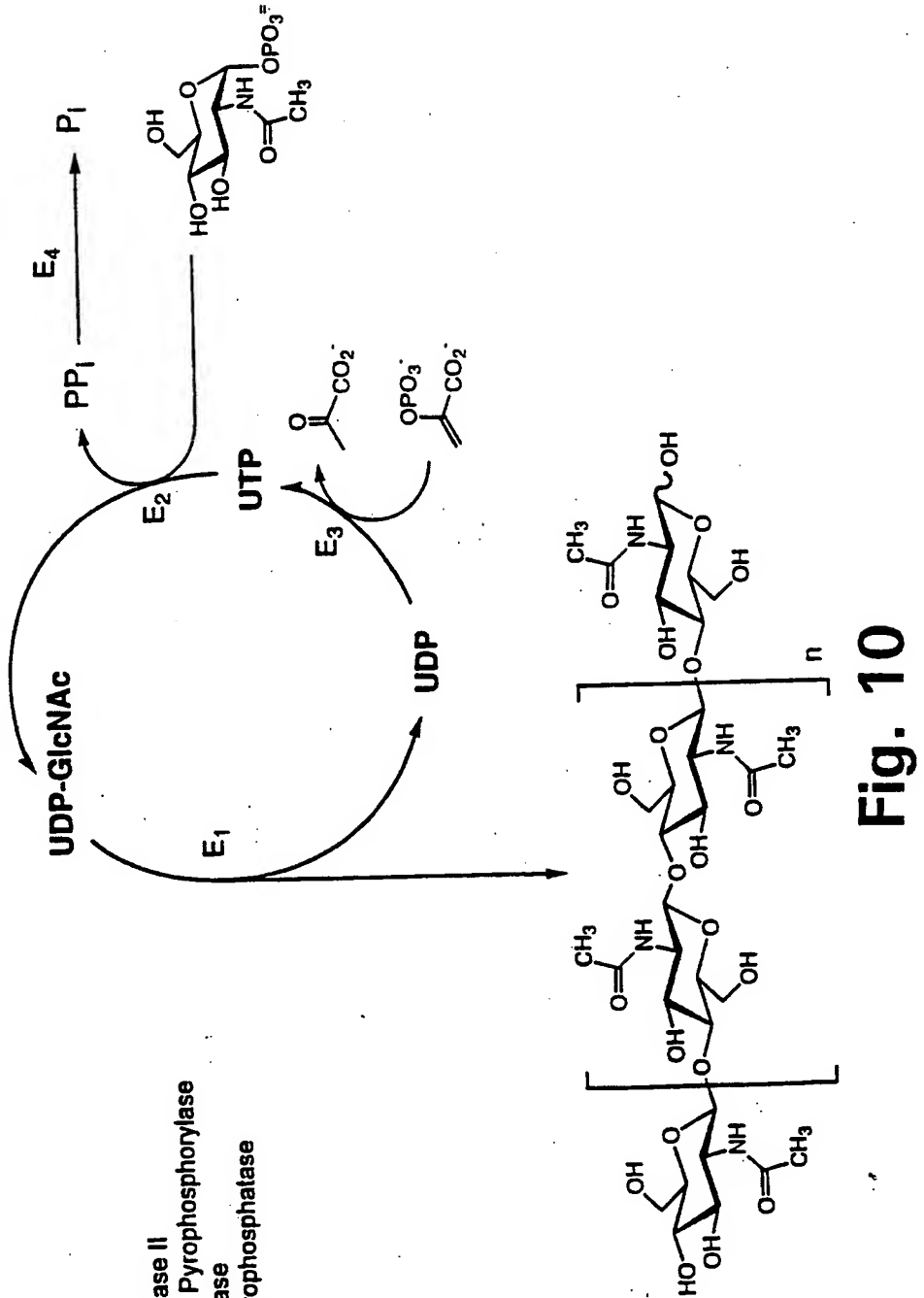


Fig. 10